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Septic systems, but not sanitary sewer lines, are associated with elevated estradiol in male frog metamorphs from suburban ponds

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ABSTRACT

Suburban neighborhoods are a dominant type of human land use. Many housing regions globally rely on septic systems, rather than sanitary sewers, for wastewater management. There is evidence that septic systems may contaminate waterbodies more than sewer lines. There is also mounting evidence that human activities contaminate waterways with endocrine-disrupting chemicals (EDCs), which alter wildlife sexual development. While endocrine disruption is often associated with intense activities such as agriculture or wastewater treatment plant discharges, recent evidence indicates that endocrine disruption is pervasive in frogs from suburban neighborhoods. In conjunction with other putative EDC sources, one hypothesis is that wastewater is contaminating suburban waterways with EDCs derived from pharmaceuticals or personal care products. Here, we measure estradiol (E2) in metamorphosing green frogs (*Rana clamitans*) from forested ponds and suburban ponds adjacent to either septic tanks or sanitary sewers. We show that E2 is highest in male frogs from septic neighborhoods and that E2 concentrations are significantly lower in male frogs from forested ponds and from ponds near sewers. These results indicate that septic tanks may be contaminating aquatic ecosystems differently than sewer lines. This pattern contrasts prior work showing no difference in EDC contamination or morphological endocrine disruption between septic and sewer neighborhoods, implying that suburbanization may have varying effects at multiple biological scales like physiology and anatomy.

1. Introduction

Residential suburban development is emerging at rates greater than human population growth (Liu et al., 2003; Theobald, 2005) and residential neighborhoods now cover over 25% of the conterminous United States (Brown et al., 2005). Often, expanding sanitary sewer services beyond well-developed urban city limits is logistically prohibitive; relatively inexpensive on-site septic tank installation promotes rapid suburban development (Harrison et al., 2012; Newburn and Berck, 2011). For freshwater ecosystems this may be problematic. In the greater Seattle area, for example, a comparison of lakes along an urbanization gradient indicated higher eutrophication in the lakes surrounded by suburban neighborhoods served by septic systems compared with those neighborhoods with sanitary sewers (Moore et al., 2003). There is also evidence that residential septic nutrient contamination yields an isotopic signature that is pervasive throughout riverine food webs, a signal that is absent in streams adjacent to suburban areas with sanitary sewer service (Steffy and Kilham, 2004). Compared to sanitary sewers, septic systems are thought to be a large non-point source of contamination to surface waters in suburban areas (Kookana et al., 2014; Rotaru and Raileanu, 2008), although there is evidence that contaminant levels do not differ between frog ponds from neighborhoods using septic systems and those with sanitary sewers (Lambert et al., 2015).

One form of aquatic contamination that has gained increasing attention over the past two decades is endocrine-disrupting chemicals (EDCs; Hayes et al., 2002; Kolpin et al., 2002; Reeder et al., 1998; Skelly et al., 2010; Smits et al., 2014; Vajda et al., 2008; Writer et al., 2010). EDC exposure can lead to abnormal endocrine function and development in aquatic vertebrates (Kloas et al., 2009). Amphibians, in particular, are valuable sentinels for studying the impacts of EDCs, both in the lab and the field (Kloas et al., 1999). In laboratory settings, EDCs can cause male frogs to

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produce testicular oocytes (TOs, intersex condition; Hayes et al., 2003; Langlois et al., 2010; Tompsett et al., 2013) and female-biased sex ratios in developing frog cohorts (Langlois et al., 2010; Pettersson and Berg, 2007). Frog populations from ponds in agricultural landscapes (Hayes et al., 2003; Murphy et al., 2006a; Papoulias et al., 2013) show the same signs of endocrine disruption as those seen in the lab. There is also emerging evidence that suburbanization promotes intersex in green frogs (*Rana clamitans*; Skelly et al., 2010; Smits et al., 2014) and has feminizing influences on sex ratios (assessed by gross gonadal morphology) of metamorphosing green frog cohorts (Lambert et al., 2015).

While the sources of EDCs in agricultural landscapes may be more evident (e.g., pesticides), the sources in suburban neighborhoods are less obvious. Recent work has documented a diversity of EDCs in suburban ponds that are absent in more natural, forested ponds (Lambert et al., 2015). While no pesticides or pharmaceuticals have been detected in suburban ponds, classical estrogens (e.g., estrone), phytoestrogens (e.g., daidzein) that are likely released by landscaping vegetation, as well as metalloestrogens that can be released from a variety of sources like runoff from roads or buildings have been detected in suburban ponds where endocrine disruption in frogs is also present (Lambert et al., 2015). There is also evidence suggesting wastewater-sourced contamination, such as pharmaceuticals or personal care products, may be entering suburban frog ponds (Smits et al., 2014). While EDCs have been shown to leach from septic systems (Swartz et al., 2006; Carrara et al., 2008) and sewer lines (Reynolds and Barrett, 2003; Rutsch et al., 2008) into groundwater, it is currently unclear whether septic systems and sanitary sewers have different effects on suburban surface water contamination (Karpuzcu et al., 2014; Smits et al., 2014). Interestingly, studies have not yet detected differences in amphibian endocrine disruption between neighborhoods on septic systems and those with sanitary sewers (Lambert et al., 2015; Smits et al., 2014). Thus far it has been challenging to infer the causes of suburban endocrine disruption, though current evidence indicates a potentially broad array of sources including yard maintenance and wastewater (Lambert et al., 2015; Smits et al., 2014). Compared to morphological endpoints, a more sensitive biological endpoint, such as direct endocrinology assays, may be useful for discerning the links between suburban landscape structure or wastewater removal mode and endocrine disruption.

Surprisingly, few studies evaluate actual hormone titers in amphibians from environments contaminated by EDCs. Because steroid concentrations can vary in response to biological factors (e.g., sex, age, or seasonality), identifying links between land use and differences in steroid concentrations requires careful study design (Guillette et al., 1999). Variation in hormones can, however, provide a more acute assessment of contamination sources that is not provided by gross morphological analyses (Guillette et al., 1999; Mosconi et al., 2005). While Murphy et al. (2006b) found no link between adult frog steroid concentrations and pesticide use in agricultural areas, Mosconi et al. (2005) found that adult male frogs in an agricultural site had lower androgen and higher estrogen levels than adult males from a more pristine site, implicating EDC contamination. In this study, we measure estradiol (E2), one of the dominant vertebrate estrogens (Bentley, 1998). We focus on both male and female froglets and control for ontogeny by sampling frogs at the same developmental stage (metamorphosis). In particular, we sampled metamorphs from forested ponds as well as ponds in suburban neighborhoods using either septic systems or sewer lines. Because we have prior evidence of morphological feminization (Lambert et al., 2015; Skelly et al., 2010; Smits et al., 2014), our goal was to explore whether a more sensitive physiological endpoint (E2) varied with wastewater removal mode as well as with suburban landscape composition.

2. Materials and methods

2.1. Site selection and characterization

Our methods for site selection are described in detail in a prior study looking at morphological signs endocrine disruption in this species (Lambert et al., 2015). We selected four forested ponds as well as eight suburban ponds (five on septic and three on sewer) that were less than 0.5 ha in surface area and were within a 35 km radius the center of New Haven, CT, USA. We chose suburban ponds such that septic systems and sewer lines were all within 20 m of the pond shorelines. We verified septic or sewer location with homeowners. For the most ideal reference sites, we selected forested ponds that were entirely forested within a 200 m buffer. Using a 2013 0.3 m resolution global satellite base map (ESRI World Imagery) in ArcMap 10.1 (ESRI, Redlands CA), we derived custom local land cover layers. We manually digitized the surrounding landscape around ponds within a 200 m radius buffer (Fig. 1), isolating forest cover, paved surfaces, building surface area, and lawn cover and calculated the proportion of each land cover category. A 200 m buffer has been successfully used to relate suburban landscape structure with frog endocrine disruption previously (Lambert et al., 2015; Skelly et al., 2010).

2.2. Frog sampling

We focused on *R. clamitans*, a species which has been widely used for endocrine disruption studies from both agricultural and suburban field studies (Lambert et al., 2015; McDaniel et al., 2008; Murphy et al., 2006a,b; Skelly et al., 2010; Smits et al., 2014). This species has a long larval period, remaining as a tadpole for approximately one year (Mintz et al., 1945) and adult females obtain sexual maturity two years after metamorphosing while adult males reach maturity three years after metamorphosis (Shirose and Brooks, 1995). While sexual differentiation occurs close to metamorphosis in most anurans (Ogielska and Kotusz, 2004), sexual differentiation in *R. clamitans* occurs within the first third of larval development, far before metamorphosis (Mintz et al., 1945), such that gonadal differentiation is completed by metamorphosis in this species (Ogielska and Kotusz, 2004).

We sampled metamorphic (Gosner stages 43–46) *R. clamitans* from each forested and suburban site. Within 24 h of capture, we euthanized frogs with tricaine methanesulfonate (MS-222). We then immediately measured snout-vent length (SVL, in mm) dissected each metamorph, assessed sex by examining gross gonadal morphology, snap froze the specimen on dry ice, and finally stored them at −80 °C. These specimens are a subset preserved from a prior study (Lambert et al., 2015). Because of their sufficiently long time-to-metamorphosis, *R. clamitans* can be sexed definitively at metamorphosis by examination of the gonad's external anatomy (Ogielska and Kotusz, 2004). Our research was conducted under approval of Yale Institutional Animal Care and Use Committee (IACUC) protocol 2012 – 10361 and Connecticut Department of Energy and Environmental Protection permit 0112019d.

2.3. Estradiol measurements

We measured whole-body 17β-estradiol levels for each metamorph following a protocol validated by Brande-Lavridsen et al. (2008) with minimal modifications. Briefly, we measured the wet mass of thawed frogs and then homogenized each frog individually using a Kinematica (Bohemia, NY) Polytron 2500E homogenizer with a PT-DA 20/2 dispersing aggregate (193 mm shaft length, 100–2000 mL volume). We homogenized each whole body with 1 mL 0.5 M sodium hydroxide and allowed the tissue to digest